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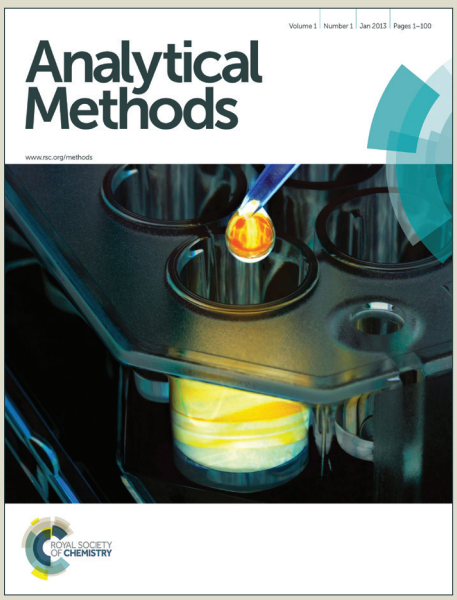

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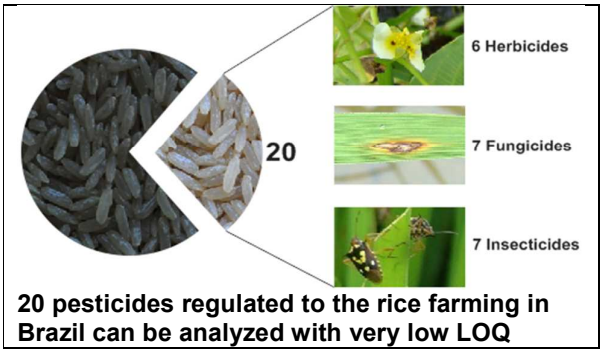

20 pesticides regulated to the rice farming in Brazil can be analyzed with very low LOQ

Determination of twenty pesticides in rice employing QuEChERs and LC-ESI-MS/MS

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Abstract

This paper describes a method for the determination of twenty pesticides in rice grains by liquid chromatography-tandem mass spectrometry with electrospray ionization in positive mode (LC-MS/MS). The QuEChERS method was used for the extraction of pesticides and clean-up of samples. Using a phenyl-based chromatographic column and a gradient of mobile phase composed by acetonitrile/water (95/5, v/v) and formic acid 0.1%, the analytical method was optimized with a total run time of 15 min. MS/MS parameters were optimized to provide higher sensitivity for each compound, resulting in limits of detection and quantification in the ranges of 0.1-17.6 ng mL^{-1} and 0.4-58.8 ng mL^{-1}, respectively. The performance of the method was also evaluated in terms of linearity, precision (instrumental, intra-assay and inter-assay), accuracy (recovery), and then it was applied to eight commercial rice samples from different suppliers. The results demonstrated the ability of the method to detect all the 20 pesticides with precision and accuracy according to the protocols established by the most important organizations and validation guidelines. Furthermore, the limits of quantification of the method were expressively lower than the maximum residue limit (MRL) established by Brazilian Health Surveillance Agency (ANVISA) for these pesticides in rice grains, which allow its application for monitoring real samples.

1. Introduction

Rice is responsible for providing on average more than 20% of energy supply and 14% of the protein source consumed by the world population. Its cultivation demands 1.5 million hectares worldwide, where 75% of the production comes from the irrigated rice type *Oryza sativa* L.[1] Brazil is the seventh largest rice producer, being the first among non-Asian ones, and most of its production is concentrated in the Southern states.[2] To ensure rice production, pesticides such as herbicides, fungicides and insecticides are widely used. Currently, the grain production represents one of the largest consumer markets for pesticides in Brazil.[3]

Several studies describe the toxicity of synthetic pesticides for human health; however they are also important to guarantee wide production, which is economically relevant for many countries. Each country establishes a maximum residue limit (MRL) that could be added on each type of food and/or beverage. Regarding the international market the limit allowed is determined by the country which is importing the product. Therefore, each country has its own regulations according to different reasons. In Brazil, since 2008 ANVISA establishes almost annually the MRL for pesticides in rice. Currently a total of 71 pesticides and their MRL are listed.[4] Based on these reasons, multi residue analytical methodologies have been developed for monitoring pesticides in foods.

Usually, pesticide residue analyses involve two steps: extraction of target analytes from the matrix and chemical separation and determination.[5,6] The QuEChERS method, characterized by being quick, easy, cheap, effective, rugged and safe, has become of the most popular extraction and/or clean-up strategies for pesticide analyses in food samples.[7] It involves, as the first step, the addition of a specific extraction organic solvent together with some additives used in order to accomplish the dryness as well as to promote the salting-out effect. The second step involves essentially the clean-up of the sample extract to eliminate interfering species such as fatty acids and chlorophyll.[8,9] This method allows modifications when applied to samples that present different characteristics[9], as in the case of beverages (juices and wines),[10,11] vegetables,[5,12] fruits,[12,13] and cereal grains,[12,14] as well as rice[15,16]. As previously

mentioned, the second step is the chromatographic separation, which can be performed by both gas or liquid chromatography.[15,17,18] However, liquid chromatography coupled to a mass spectrometry in tandem (LC-MS/MS) is the preferred one for determination of pesticides in food products, especially because it offers high sensitivity and selectivity with no needs of derivatization,[19] being particularly adequate for thermolabile and non-volatile compounds. Furthermore, several studies have reported the successful association between QuEChERS method and LC-MS/MS analysis to determine pesticide residues in different food samples.[20–22]

In this study, the validation of a simple, sensitive, reliable, efficient and rapid method using extraction by modified QuEChERS followed by LC-ESI-MS/MS for the determination of twenty residues of pesticides in rice is described. This study sought developing a method able for determining compounds frequently used in rice crops in Brazil, allowing its application in the monitoring of a relatively wide range of pesticides.[4]

2. Experimental

2.1 Chemicals: All analytical standards of pesticides (azoxystrobin, carbendazim, carboxine, cyclosulfamuron, cycloxidim, cyproconazole, clomazone, chlorantraniliprole, epoxiconazole, ethoxysulfamuron, imidacloprid, metsulfuron-methyl, mycrobutanil, oxadiazon, paraoxon-methyl, pirimiphos-methyl, thiabendazole, thiamethoxam, thiobencarb and tricyclazol, purity >98%), and sulfametoxazol (purity >98%) were supplied from Sigma Aldrich (São Paulo, SP, Brazil). HPLC grade methanol, acetonitrile and formic acid (49-51% (T)) were obtained from Merck (Darmstadt, Germany). Anhydrous magnesium sulfate (99.8%), anhydrous sodium acetate (99%) and PSA 40µm (Agilent, USA) were purchased from J.T. Baker (Tokyo, Japan). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Solutions: The stock solutions of all pesticides and sulfametoxazol (used as surrogate standard) were prepared separately (1000 mg L^{-1}) in methanol or acetonitrile and stored at -4°C. From the stock solutions, a mixture of all pesticides containing different concentrations based on the MRL of each one

was prepared in water. It was used for preparing the working standard solutions also in acetonitrile, including the analytical standards and also for spiking the blank matrix extract.

2.3 Blank control: To develop the present method, samples of rice grains obtained from EPAGRI were used as blank control. Both the cultivation and harvesting were rigorously monitored by professional workers for ensuring that the matrixes were free of pesticides. The grains were harvested, peeled, and ground resulting in particles with 0.2-1.0 mm which were selected for all studies.

2.4 Sample preparation using modified QuEChERS method[9] (Figure 1): For the extraction, 14 mL of acetonitrile with 1.0% acetic acid and 1.0 mL of a solution containing sulfametoxazol (142 mg L^{-1}, as surrogate standard) were added into a 50 mL PTFE tube containing 5 g of sample previously ground (see sessions 2.3 and 2.7). After 30 min. kept interacting, 2.0 g of anhydrous magnesium sulfate and 0.5 g of sodium acetate were added into the mixture, vortex stirred for 1.0 min and then centrifuged at 4,000 rpm for 1.0 min. To perform the clean-up 1.5 mL of the liquid phase was extracted, placed in a 15 mL falcon tube, in which were added 150 mg of anhydrous magnesium sulfate and 50 mg of PSA (primary and secondary amines). The falcons were agitated in vortex for 1.0 min and centrifuged for 1.0 min at 4,000 rpm. 1.0 mL was then collected from the supernatant, which was placed directly into vials for automatic injection into the chromatographic system. All procedures were performed in triplicate.

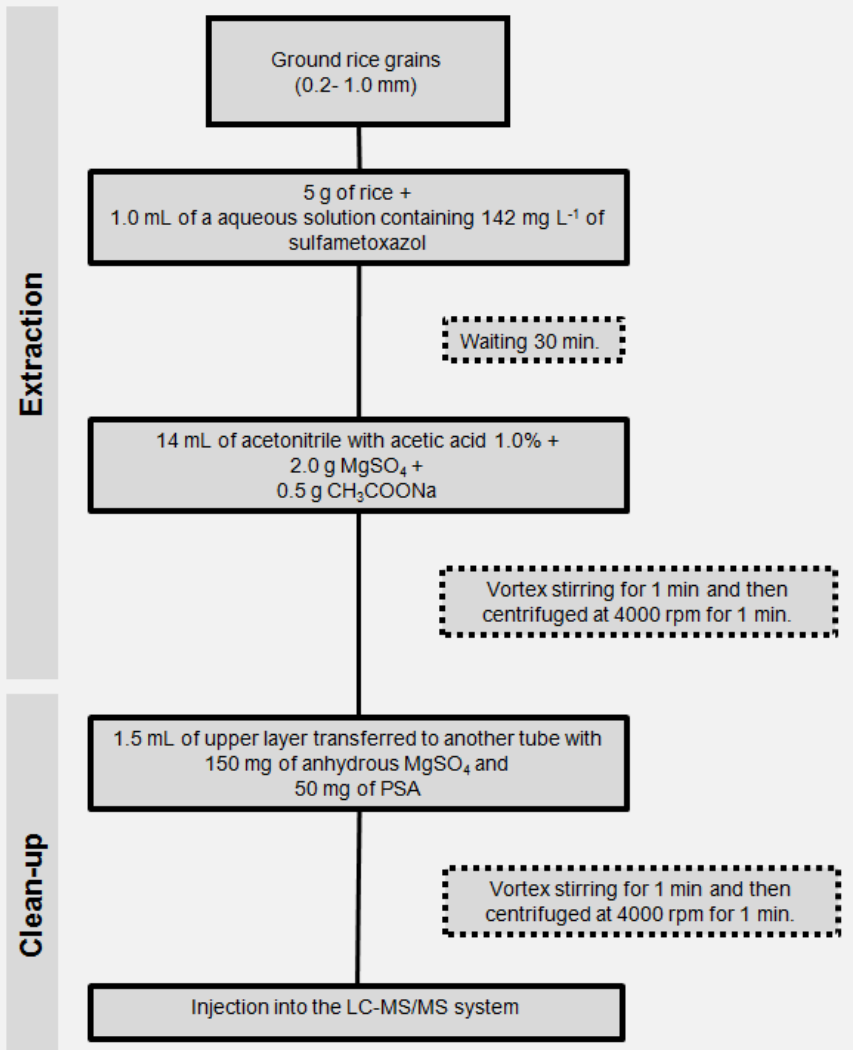

Figure 1. Flowchart of the QuEChERS procedure applied in the sample preparation.

2.5 Analytical curves: External standard analytical curves were plotted in seven levels of concentration (see Table 2 for the linear ranges), which were obtained by dilution of the mixture containing all analytes. Three replicates of all working standard solutions (1.0 mL each) were prepared in acetonitrile.

2.6 Evaluation of the method: The proposed method was evaluated in terms of linearity (slope of the external standard analytical curves and their determination coefficients – R^2), precision (instrumental, repeatability (intra-assay) and inter-assay) for the intermediate concentration of each linear range, limits of detection (LOD) and quantification (LOQ) obtained from the signal to noise ratio, 3:1 and 10:1, respectively,[23] and accuracy. To evaluate the accuracy, recovery assays using grains of rice in the absence of pesticides (blank control) were used.[24,25] This procedure was performed by addition of the surrogate standard (final concentration of 9.5 mg L^{-1}) and four concentration

levels of each analyte in 5 g of rice, before the addition of acetonitrile and salts used in the QuEChERS extraction. The four concentrations used in the recovery assays represented the entire linear ranges.

2.7 Applicability of the method: The proposed method was finally applied in the determination of 20 pesticides in eight commercial rice samples provided by different suppliers. All samples were treated as the blank control: ground resulting in particles with 0.2-1.0 mm which were selected for all studies. The modified QuEChERS method previously described was applied and samples were then analysed using the proposed LC-MS/MS method.

2.8 Instrumentation and methodology (LC-MS/MS): All analyses were performed on an Agilent HPLC series 1200 system, equipped with a quaternary pump, a membrane degasser and an auto-sampler (Agilent Technologies, Palo Alto, CA). Separation was carried out on a Synergi Polar RP column, 150 mm × 2.0 mm (150 mm, 2.0 mm i.d., 4 µm particle size, Phenomenex). The mobile phase used was composed by acetonitrile/water (95/5, v/v) as solvent **A** and formic acid 0.1% as solvent **B**, using the gradient mode as follows: 0-1 min, 20% solvent **A**; 1-10 min, 20% to 90% of solvent **A**; 10-12 min, 90% solvent **A**; 12-12.01 min, 95% to 20% of solvent **A**; 12.01-15 min, 20% of solvent **A**. The column was kept at 40°C and the flow rate of the mobile phase was 400 µL min^{-1}. The injection volume was 10 µL. The LC was coupled to a MS system consisting of a hybrid triple quadrupole/linear ion trap mass spectrometer QTrap 3200 (Applied Biosystems/MDS Sciex, Concord, Canada). The Analyst software version 1.5.1 was used for the LC-MS/MS system control and data analysis. The experiments were performed using a Turbo Ion Spray source (electrospray-ESI) in positive ion mode. The capillary needle was maintained at +5500 V. MS/MS parameters: curtain gas (N_2), 10 psi; temperature, 600°C; gas 1 (Ar), 18 psi; gas 2, off; CAD gas (N_2), high. The analytes were monitored and quantified using multiple reactions monitoring (MRM) and the MS was optimized by the direct infusion of solutions containing each analyte investigated in the present study.

3. Results and Discussion

3.1 Parameters of modified QuEChERS method: before establishing the parameters of the QuEChERS method applied to samples preparation, some previous studies were performed. In general, a slurry composed by dry samples and water is prepared before the QuEChERS extraction to make sample pores more accessible to the extraction solvent.[26] However, there are some studies reporting the use of ground rice rather than the whole grain, without any slurry preparation.[27,28] This latter approach was chosen in this work to avoid dilution of the sample and also because during grind procedure the sample is open and its surface area is slightly increased, which likely improve the extraction performance.

Considering that ethyl acetate, acetone, methanol and acetonitrile are the most used solvents for extraction in QuEChERS, ethyl acetate and acetone were initially discarded due to the degradation of some compounds, even after acidification.[29] Acetonitrile, the extractor solvent used in the original QuEChERS, was chosen instead of MeOH due to its availability and lower toxicity. To avoid pesticides degradation, acetic acid was needed in the extractor medium. Still in the extraction step, high amounts of magnesium sulfate were needed to dry the system and also for helping adsorption of non-polar compounds due to the slight increase of temperature.[8] Sodium acetate was also added into the medium for providing *salting out* effect and for buffering the solution at pH between 4-5, which is important to avoid the degradation of some compounds under strongly acid conditions and also to decrease extraction of fatty acids.[30] For the clean-up step, magnesium sulfate was added in lower amounts to retain the remaining water. Other additives can be used in this step to eliminate other specific compounds from the matrix. In general, C18, GCB (Grafitized Carbon Black) and/or PSA are used. C18 is added in case of samples having more than 2% of fat[30,31], while GCB is useful for samples rich in chlorophyll; PSA can eliminate organic and fatty acids in low concentrations, besides having shown success in eliminating sugar and phenolic compounds as well.[11] Considering that rice grains do not pose much over 2% of fat[32], C18 was not used in this work. Addition of GCB was tried, however the recovery values were low, due to its ability to interact with planar structures such as

carbendazim, imidacloprid, thiabendazole and tricyclazol through π-π, ionic and/or hydrophobic interactions[33–35]. Thus, PSA was the only additive used during clean-up step besides magnesium sulfate, providing recovery values in acceptable range, as it is shown as follows.

To evaluate errors arising from the extraction process, sulfametoxazole was used as surrogate standard. It was added into the sample before the extraction procedure has been started. It was chosen because of its structural similarities with some analytes eluting in similar retention times during the chromatographic separation, for not being found in rice samples, and also because of its easy detection by ESI-MS in positive mode.

3.2 LC-MS/MS parameters

Considering the high amount of pesticides monitored in this work and also the complexity of the sample, the MS/MS system and MRM mode for detection were used to provide high specificity for the method. The MRM allows the detection of both the parent ion and one of its known fragments. In addition, using the MS/MS system it is possible to monitor the products from the secondary fragmentation, which enables a much better discrimination of the interfering matrix than the use of the products of primary fragmentation (MS). To provide higher sensitivity for the method the optimum collision energy for each compound was selected aiming of getting the best signal intensity, with the best reproducibility, for each monitored fragmentation. The MRM transition that provides the high signal intensity was chosen for quantification. These optimized parameters are listed in Table 1.

Table 1 MS/MS parameters optimized for each analyte

Pesticides	MRM transition (m/z)		DP	CE (eV)		EP	CEP	CXP (V)		Dwell Time
	QIT (m/z)	CIT (m/z)	(V)	QIT	CIT	(kV)	(kV)	QIT	CIT	(ms)
Azoxystrobin	404.03>372.10	404.03>329.10	246	19	31	4.5	34.0	6	6	10
Carbendazim	192.10>160.00	192.10>132.10	36	23	39	5.0	12.0	4	4	50
Carboxine	236.14>143.10	236.14>43.10	36	19	51	4.0	14.0	4	6	10
Cyclosulfamuron	422.03>260.90	422.03>218.00	31	21	31	5.0	18.0	6	4	10
Cycloxidim	326.11>280.10	326.11>180.00	31	17	25	4.0	16.0	6	4	10
Cyproconazole	293.09>70.00	293.09>125.10	36	35	37	4.5	14.0	4	4	10
Clomazone	241.15>126.00	241.15>125.00	36	25	25	7.0	14.0	4	4	10
Chlorantraniliprole	483.85>452.90	483.85>286.00	161	21	23	6.0	20.0	6	6	10
Epoxiconazole	331.06>101.10	331.06>102.10	61	67	69	4.5	16.0	4	4	10
Ethoxysulfamuron	399.05>260.70	399.05>218.00	41	23	39	2.5	34.0	4	4	10
Imidacloprid	257.09>210.10	257.09>176.00	26	17	19	6.5	16.0	4	4	10
Metsulfuron-methyl	382.10>167.00	382.10>141.00	31	19	21	5.5	28.0	4	4	10
Mycrobutanil	290.04>70.00	290.04>125.00	41	37	39	4.5	14.0	4	4	10
Oxadiazon	346.06>304.00	346.06>184.90	31	17	37	6.5	28.0	6	4	10
Paraoxon-methyl	248.02>202.00	248.02>109.00	46	19	35	6.5	14.0	4	4	10
Pirimiphos-methyl	305.93>108.10	305.93>164.20	21	41	23	12.0	16.0	4	4	10
Thiabendazole	202.08>175.00	202.08>131.00	56	33	43	4.0	10.0	4	4	50
Thiamethoxam	292.03>211.10	292.03>132.10	26	15	27	3.5	16.0	6	4	50
Thiobencarb	259.05>126.00	292.03>125.00	26	21	21	5.5	14.0	4	4	10
Tricyclazol	190.08>136.10	190.08>162.90	51	35	31	10.0	12.0	4	4	10
Sulfametoxazole	254.02>156.00	254.02>108.10	31	19	33	5.5	14.0	4	4	10

Legend: QIT: Quantitation ion transition; CIT: Confirmation ion transition; DP: *de-clustering potential*; CE: collision energy; EP: *entrance potential*; CEP: *collision cell entrance potential*; CXP: *collision cell exit potential*.

The dwell time parameter was also optimized for each transition. As can be seen in Table 1, a dwell time of 10 ms was adequate for the most analytes, however 50 ms was necessary for scanning carbendazim, thiabendazole and thiamethoxam. The influence of dwell time for detection of carbendazim is shown in Figure 2.

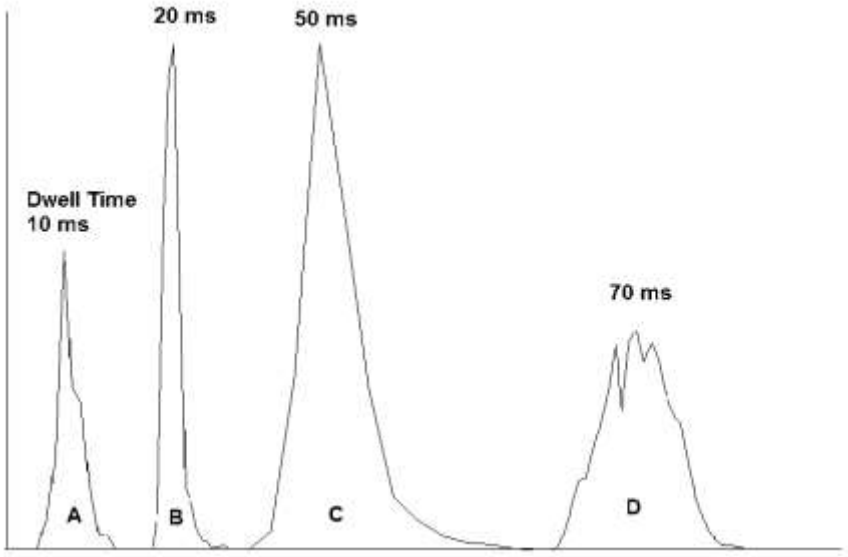

Figure 2. Carbendazim related peaks obtained in different dwell times, through monitoring quantification ion transition (QIT = m/z 160). The peaks A, B, C and D present the same retention time and are grouped in only one graph to facilitate the comparison of peak shapes.

As it can be seen in Figure 2, a long dwell time resulted in splitting of the peak, while a much-reduced time resulted in low detectability. For this reason 50 ms was considered adequate for providing both a symmetric peak and high detectability.

For the chromatographic separation, the Synergi Polar RP column was chosen due to its high polarity and selectivity for compounds that present aromatic rings on their structures, which are characteristic of many pesticides. This affinity is provided from π-π interactions between analyte-stationary phase, and the polar end-capping present in the column increases the retention of polar compounds. Still considering the high amount of analytes determined in this work and the structural similarities of some, a gradient of the mobile phase was needed for providing satisfactory separation. Firstly, the mobile phase was operated as follows: 0-1 min, 5% solvent **A**; 1-15 min, 5% to 95% of solvent **A**; 15-25 min, 95% solvent **A**; 25-30 min, 95% to 5% of solvent **A**, using a flow rate of 200 μL min^{-1}. These conditions provided an analysis time higher than 25 minutes.

Gradient of the mobile phase and also the flow rate were adjusted for achieving smaller run time with negligible overlapping peaks considering MS/MS detection. The optimized conditions allowed the analysis of all pesticides in around 12 minutes, according to Figure 3.

Figure 3. Chromatograms in MRM mode containing the transition monitored in the quantification (QIT) of the pesticides.

3.3 Evaluation of the developed method

3.3.1 Figures of merit: The performance parameters of the proposed LC-MS/MS method are presented in Tables 2 and 3.

Table 2 Performance parameters of the method evaluated for all pesticides.

Pesticides	Linear range (µg mL^{-1})	Precision (RSD)[a]			Linearity	Recovery[b]	
		Inst	Rep	Inter		mean %	RSD %
Azoxystrobin	0.0047-0.0330	5.05	5.20	5.85	y = 8.626x -0.001 R^2 = 0.9913	83.1	7.58
Carbendazim	0.0226-0.1582	2.68	5.82	5.17	y = 16.218x +0.134 R^2 =0.9973	86.9	13.0
Carboxine	0.0101-0.0707	4.50	9.61	8.28	y = 6.117x +0.014 R^2 = 0.9909	105.3	11.6
Cyclosulfamuron	0.0238-0.1666	9.60	11.3	9.20	y = 1.003x R^2 = 0.9950	95.0	13.6
Cycloxidim	0.0208-0.1456	5.53	5.71	5.04	y = 2.299x -0.003 R^2 = 0.9989	111.8	6.76
Cyproconazole	0.0019-0.0134	13.9	13.6	12.6	y = 1.407x R^2 = 0.9990	101.9	4.40
Clomazone	0.0040-0.0280	6.37	13.1	10.7	y = 1.215x -0.004 R^2 = 0.9958	96.3	16.2
Chlorantraniliprole	0.0343-0.2403	7.89	8.31	7.58	y = 0.857x -0.006 R^2 = 0.9981	89.4	14.0
Epoxiconazole	0.0238-0.1663	6.13	15.8	12.6	y = 0.672x -0.009 R^2 = 0.9976	74.3	3.80
Ethoxysulfamuron	0.0238-0.1666	4.42	4.20	5.60	y = 1.412x -0.005 R^2 = 0.9974	96.3	12.4
Imidacloprid	0.0588-0.1595	9.58	8.03	10.4	y = 0.003x -0.002 R^2 = 0.9955	104.2	15.5
Metsulfuron-methyl	0.0210-0.1470	4.41	7.02	7.72	y =5.529x -0.018 R^2 = 0.9990	100.3	17.6
Mycrobutanil	0.0264-0.1778	4.63	8.17	6.79	y =1.233x -0.012 R^2 = 0.9970	96.3	8.74
Oxadiazon	0.0401-0.2071	5.83	6.66	5.61	y =1.096x -0.001 R^2 = 0.9913	87.2	12.9
Paraoxon-methyl	0.0205-0.1456	4.89	6.35	6.14	y =1.046x -0.002 R^2 = 0.9958	98.5	10.7
Pirimiphos-methyl	0.0390-0.2730	5.69	5.39	5.58	y = 14.506x -0.077 R^2 = 0.9998	75.3	3.51
Thiabendazole	0.0101-0.0707	3.42	2.06	3.51	y = 2.676x -0.001 R^2 = 0.9992	92.6	7.34
Thiamethoxam	0.0403-0.2820	4.61	4.35	4.76	y = 1.044x +0.008 R^2 = 0.9912	105.5	5.29
Thiobencarb	0.0065-0.0176	12.4	13.7	12.7	y = 0.531x R^2 = 0.9928	103.0	4.60
Tricyclazol	0.0256-0.1722	6.67	8.92	7.33	y = 2.916x +0.011 R^2 = 0.9922	113.8	4.18

[a] Performed using the intermediate concentration of each studied linear range; [b] performed in four concentration levels of each analyte, representing the entire linear ranges.
Legend: Inst, Rep and Inter are instrumental, repeatability and inter-assay precisions, respectively; RSD = relative standard deviation.

Table 3 Limits of detection (LOD) and limits of quantification (LOQ) of the proposed method and Maximum Residue Limits (MRL) of the pesticides in rice, according to ANVISA.[36,37]

Pesticides	MRL (μg g^{-1})	LOQ (μg mL^{-1})[a]	LOD (μg mL^{-1})[a]
Azoxystrobin	0.100	0.0018	0.0005
Carbendazim	0.500	0.0028	0.0008
Carboxine	0.200	0.0021	0.0006
Cyclosulfamuron	0.500	0.0087	0.0026
Cycloxidim	0.500	0.0033	0.0010
Cyproconazole	0.030	0.0019	0.0006
Clomazone	0.100	0.0028	0.0008
Chlorantraniliprole	0.500	0.0134	0.0040
Epoxiconazole	0.300	0.0093	0.0028
Ethoxysulfamuron	0.500	0.0074	0.0022
Imidacloprid	0.500	0.0588	0.0176
Metsulfuron-methyl	0.500	0.0033	0.0010
Mycrobutanil	0.500	0.0264	0.0079
Oxadiazon	0.500	0.0401	0.0120
Paraoxon-methyl	0.500	0.0205	0.0062
Pirimiphos-methyl	10.000	0.0004	0.0001
Thiabendazole	0.200	0.0069	0.0021
Thiamethoxam	1.000	0.0164	0.0049
Thiobencarb	0.050	0.0065	0.0020
Tricyclazol	0.500	0.0256	0.0077

[a] LOQ and LOD calculated by the signal to noise ratio, 10:1 and 3:1, respectively.

Linearity and Precision: The coefficient of determination (R^2) for each standard curve was higher than 0.99 demonstrating the linearity of the method for all compounds. The instrumental precision values (n = 10) obtained for peak areas were evaluated for the intermediate concentration level of the standard curves prepared for each analyte. As it can be seen in Table 2, they were less than 10% for the most analytes, except for the cyproconazole and thiobencarb, which shown instrumental precisions of 13.9 and 12.4, respectively. To evaluate the repeatability (or intra-assay precision) of the method, the same concentration of each compound (intermediate level of the linear ranges) was prepared 8 times (n = 8) by the same analyst and each solution was injected in duplicate. RSD values obtained for this assay ranged from 2.1% to 15.8% for thiabendazole and epoxiconazole, respectively. The inter-assay precision was

also evaluated (n = 8) for the method, and the results didn't exceed 13%. Epoxiconazole was the only compound that presented a relative standard deviation slightly higher than 15% in the precision assays. This particular result is over the limit accepted by ANVISA (15%), however, according to the most organizations and validation guidelines, this result can be accepted taking into account the studied concentration levels and the complexity of food samples.[38–42]

Limits of detection and quantification: Table 3 shows values obtained for LOD and LOQ of the method and also the maximum residue limits (MRL) established by ANVISA for each studied pesticide in rice.[36,37] As can be seen, the present LC-MS/MS method provided LOQ values between 2-74 ng mL^{-1} and LOD between 1-22 ng mL^{-1}, or 0.002-0.074 μg mL^{-1} and 0.001-0.022 μg mL^{-1}, respectively. Considering the sample preparation, where 5 g of rice were treated in a total volume of 15 mL, all LOD and LOQ are low enough to allow the determination of all pesticides in limits lower than the MRL established by ANVISA. Thus, for the most compounds it is possible to detect and quantify samples with pesticides concentrations between 9-199 and 3-60 times lower than the MRL, respectively. However, the pirimiphos-methyl can be detected and quantified in concentrations 31,000 and 9,300 times lower due the high MRL established by ANVISA (See Table 3). These results clearly indicate that the proposed method is suitable for monitoring the twenty aforementioned pesticides in rice samples. Considering the low LOQ for the most compounds, it is possible to use standard curves in lower concentrations than the presented in this study.

Accuracy (recovery): Due to the complexity of the sample, recovery assays were performed in order to observe the matrix effect in the quantification of the analytes. For this, four analyte concentration levels were added (0.004-0.07 μg mL^{-1}, varying for each analyte according to the linear range) into the sample. Table 2 presents the average recovery values, expressed as percentage, obtained for each concentration level and their respective RSD%. Values from 74% to 114% with RSD lower than 18% were obtained, which are in the acceptable range of recovery for trace residue analysis, (usually between 70% and 120%, with RSD ±20%).[43,44]

3.4 Application

A total of eight commercial rice samples provided by different brands were analysed in this work. As previously described, all samples were prepared using the modified QuEChERS method and then they were directly injected into the LC-MS/MS system without further dilution. Mycrobutanil and pirimiphos-methyl were detected in all samples, however only one presented pirimiphos-methyl in concentration higher than the LOQ, which was quantified in 0.014 μg mL^{-1}, using standard addition calibration. This concentration corresponds to 0.042 μg g^{-1} considering the sample preparation (around 240 times below the MRL for this compound). Epoxiconazole was also detected in two samples, however also in concentrations below the LOQ. All other pesticides were present in concentration lower than the LOD. Summarizing, the results demonstrated that all samples are in agreement with the concentrations allowed by ANVISA (below the MRL). Results found in this work are in agreement with other recent studies that report determination of pesticides in different food samples, which demonstrated that the most analyses performed have presented concentrations below the established MRL.[5,5,45] These results are indicative of good agricultural practice in the studied area[5], explained by the rigorous regulation and monitoring system in worldwide, including Brazil, and also consumer demands.

Several works in literature report the determination of pesticides in different matrices, especially in food. However, to the best of our knowledge most of the multi residue analytical methods reported are just partially applied in the determination of pesticides used in food farming, which in this particular case have MRL established by ANVISA.

For example, in a method reported for determination of 203 compounds in rice grains using CG-MS, high specificity and low LOQ were achieved in around 30 minutes, but only eleven of the total analytes have MRL established by the same agency.[46] A second work reported the determination of a total of 98 compounds among organophosphorous and carbamates by LC-MS/MS in less than 20 minutes, but only 10 are actually regulated, including pirimiphos-methyl and thiobencarb, which can be also detected for the present method.[27] In a third study reported in 2013, 124 residues were determined by GC-MS/MS in around

45 minutes. Fourteen of the total analysed pesticides are allowed by ANVISA regulation, including difenoconazol, microbutanil and pirimiphos-metil.[15]

In the proposed method, all the 20 pesticides analyzed are regulated by ANVISA and must be in compliance with the established MRL. In addition, all of them presented LOQ significantly lower than the allowed limits with high precision and accuracy. These characteristics demonstrate the applicability of this method in the routine practice by any laboratory including those of Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) that attend the Program on Pesticide Residue Analysis in Food (PARA).

4. Conclusions

The method developed and described in this manuscript was linear, precise and accurate, according to most important organizations and validation guides. The use of MS/MS provided higher selectivity and sensitivity for the method, providing reliable results with LOD and LOQ values lower than the MRL established by ANVISA. It pronounces the successful application of using QuEChERS and LC-MS/MS in association to determine pesticides in food samples. The validated method is proposed as alternative for monitoring of twenty pesticides residues in rice, all them used in real cultivation of this grain. The relative low volume of organic solvents and short analysis time needed by the method, make it interesting for routine analysis, quality control and monitoring performed by ANVISA on its Program on Pesticide Residue Analysis in Food (PARA).

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